1954

Preliminary characterization of a crystalline fraction from Pteryxia terebinthina (Hook.) Coult. & Rose var. terebinthina

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THE PRELIMINARY CHARACTERIZATION OF A CRYSTALLINE FRACTION FROM *Pteryxia terebinthina* (Hook.) Coult. & Rose var. *terebinthina*

by

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B. S., Montana State University, 1949

Presented in partial fulfillment
of the requirements for the degree of
Master of Science in Pharmacy

MONTANA STATE UNIVERSITY

1954

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June 3 1954
Date

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ACKNOWLEDGMENT

The author is indebted to Dean Jack E. Orr for his assistance and guidance throughout this work and to Professor Tracey G. Call and Dr. J. M. Stewart for many helpful suggestions.

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TABLE OF CONTENTS

HISTORICAL

The medicinal value of certain plants found recognition very early in the history of all known peoples and evidence seems to indicate that this discovery occurred long before man had learned to write. The Emperor Shen Nung, who lived about 2700 B.C., is credited with observing the antifebrile effects of Ch'ang Shang which has been shown to contain antimalarial alkaloids. He also noticed the diaphoretic and stimulatory effects of the drug Ma Huang, from which the active alkaloid ephedrine has been isolated only recently. The Ebers Papyrus, which was discovered in 1873 and written about 1500 B.C., is one of the earliest references to the medicinal uses of plants. It describes more than 700 herbal remedies, including many that are familiar today such as poppy, castor oil, squill, aloe, and caraway. Thus the use of plants for the treatment of disease dates back at least five thousand years.

Modern scientific developments have greatly changed the methods of medicine and have introduced new drugs which are unknown in nature, but drugs derived from plants are still finding extensive and, in some cases, increasing use. Quinine, from the bark of Cinchona officinalis L., has been used for over three centuries and is still one of the most important drugs today for treating malaria. From this bark also is obtained quinidine, which is valuable in the treatment of auricular fibrillation. Plants of the Apocynaceae, Liliaceae, Ranunculaceae and Scrophulariaceae families are sources of the well known cardiac glycosides such as digitalis, strophanthin, and many others. Ergot, opium, cocaine, curare, the belladonna alkaloids, veratrum viride, khellin and rutin,

to mention only a few, are drugs which are derived from plants and extensively used in medicine today. The lower plants are sources of many of today's newer antibiotics. Penicillin, for example, is derived from the mold Penicillium notatum. Streptomycin, aureomycin, neomycin, terramycin and many other antibiotics are derived from the actinomycetes, which are the non-motile organisms intermediate between the bacteria and the molds. These are found in soils, river and lake bottoms and on the surfaces of plants.

THE UMBELLIFERAE

The Umbelliferae or Carrot family consists of about 270 genera and 2700 species of herbs which are widely distributed, being most abundant in the temperate zone. Some of the Umbelliferae such as carrots, parsley, parsnip and celery have found use as foods. Caraway, dill, coriander and anise have been used as condiments. The Indians of western North America used many of the roots as food and mention is made of the fleshy roots in many of the early reports of western exploration. Rosenthal,[15] speaking of Cymopterus glomeratus (Nutt.) Raf. (C. acaulis) reports the root as a favorite food of the Pawnee Indians. Cymopterus Fendleri Gray was mentioned by Thurber[29] as the "Cimaja of the Mexicans at Santa Fe and by them used to flavor meats and make bitters for liquor". Torrey[30] mentions the use of Cymopterus montanus (Nutt.) T. & G. (Phellopterus montanus) by the Mexicans of the southwest who call it the "Camote" or "Camote" (sweet potato). Bois[1] refers also to this plant with its fusiform roots somewhat resembling parsnip, but more tender and sweeter.

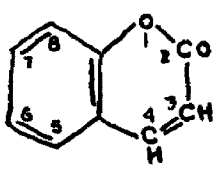
The Umbelliferae have yielded some substances of medicinal importance. Apiol, which is a white, crystalline material obtained from

Petroselinum sativum Hoffman has been used as a diuretic, stimulant, antiperiodic and emmenagogue. Anise, fennel, caraway, coriander and celery fruit have been used extensively in the past as aromatics, stomachics and carminatives. The juice of conium, or poison hemlock, entered into the famous hemlock potion of the Greeks and was used by them in putting criminals to death. Coniine, a liquid alkaloid of conium, was formerly used as an antispasmodic, sedative and anodyne, but today has fallen into almost complete disuse. Asafetida or Gum Asafetida has been used as a stimulant, expectorant, antispasmodic and laxative.

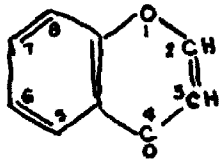
THE COUMARINS

Over a period of many years, Dr. Ernst Späth and his co-workers isolated, investigated and elucidated the chemical structures of many crystalline substances from umbelliferous plants. At least twenty-two[20] of these crystalline substances have been shown to be coumarins.

The fusion of a pyrone ring with a benzene nucleus gives rise to a class of heterocyclic compounds known as the benzopyrones. Two distinct types of benzopyrones are recognized: the benzo-α-pyrones, commonly called the coumarins, and the benzo-γ-pyrones, commonly called chromones. These two type differ from each other only in the position of the carbonyl group in the heterocyclic ring.



Benzo-α-pyrone
or coumarin

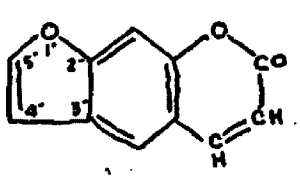


Benzo-γ-pyrone
or chromone

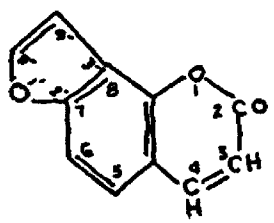
Representatives of these groups of compounds are found to occur in the vegetable kingdom, either in a free or combined state. Coumarin, which is the parent substance of the benzo-α-pyrone group, was first isolated from the tonka bean in 1820.

As Späth has shown, the Umbelliferae are rich sources of naturally occurring coumarins. Umbelliferone, which is 7-hydroxycoumarin, has been found in the free state and also has been obtained by dry distillation of umbelliferous resins, such as that of Ferula Assa-foetida L., Dorema ammoniacum Don., and Ferula galbaniflua Bois. & Buhse. Osthol, osthenol, ostruthin and ammoresinol belong to a group of hydroxy- and methoxy-coumarins with alkyl or alkylene groups attached to the benzene ring of the coumarin nucleus. All except ammoresinol, which is the main constituent of ammoniac resin of Dorema ammoniacum Don. are present in the root of Imperatoria ostruthin L., now known as Peucedanum ostruthin Koch[31]. Osthol has also been isolated from the seed of Hseh Tsuang, which is the Chinese name of a drug plant of the Umbelliferae, the seed of which has been reported to have been used for centuries in China as a tonic for sexual exhaustion.[13] The chemical constitutions of osthol,[26] ostruthin[25] and osthenol[20] have been elucidated.

Furanocoumarins, in which a furan ring is fused to the benzene ring, have been found among the Umbelliferae. Depending upon the position of fusion of the furan ring, several isomers are possible, two of which have been found to occur in nature. These two are angelicin and psoralene.[16]



Psoralene



Angelicin

The ring system of angelicin is present in isobergaptene and pimpinellin, while that of psoralene is found in bergaptene, xanthotoxol, isopimpinellin, imperatorin and isoimperatorin, oxypeucedanin and ostruthol.

The chemical structure of angelicin was determined by Späth[19] to be furano-2',3',7,8-coumarin, and is found in the plant Angelica archangelica L. Xanthotoxol, which has a hydroxyl group at position 8 of the psoralene nucleus, is also present in the roots of Angelica archangelica L. This phenolic furanocoumarin yields the known compound xanthotoxin on methylation, so its constitution is established beyond doubt.[16]

Bergaptene, the 5-methoxy derivative of psoralene, is found in the fruits of Seseli indicum (Wall) W. & A., Ligusticum acutilobum S. & Z. and Heracleum sphondylium L.[20]

5-methoxyangelicin, known as isobergaptene, and pimpinellin and isopimpinellin, both of which are dimethoxyfuranocoumarins, are found in the roots of Pimpinella saxifraga L.

The rhizome of Peucedanum ostruthium Koch, mentioned previously as a source of osthol, osthenol and ostruthin, has yielded a number of complex furanocoumarins. Among those whose chemical structure has been elucidated are imperatorin,[22] isoimperatorin,[23] oxypeucedanin[24] and ostruthol.[21] These compounds possess a saturated or unsaturated side chain attached to the benzene ring of the furanocoumarin nucleus.

Other crystalline substances which have been isolated from the Umbelliferae are the furanocoumarins with substituents on the furan ring. Peucedanin, present in the roots of Peucedanum officinale L., a European

plant but now found in North America, has an isopropyl group at the 5'-position and a methoxy group at the 4'-position of the furan ring of the psoralene nucleus. Oreoselone, also found in the same plant, has an identical structure to peucedanin except that the methoxy group is replaced by a ketone oxygen at the 5'-position. Peucedanin is the enolic methyl ether of oreoselone and can be easily hydrolyzed to oreoselone.

The roots of Heracleum sphondylium L. have yielded two crystalline substances, sphondin, whose structure has been determined,(28) and sphondylin, which is of undetermined structure but probably the isomer of sphondin.

Ammi visnaga L., a perennial herbaceous plant found in the waste lands of the Eastern Mediterranean, and a close relative of this plant, Ammi majus L., both belong to the family of Umbelliferae. Several crystalline substances have been isolated from these plants. From Ammi visnaga L. have been isolated the furanochromones khellin, visnagin and khellol glucoside, and from Ammi majus L. the furanocoumarins xanthotoxin, imperatorin and bergaptene.(12)

The coumarins and chromones have been found to be physiologically active in animals as well as man, but a review of the literature revealed that with the exception of the furanochromone khellin, the many benzopyrones isolated from the Umbelliferae have had no widespread acceptance in medicine.

Wasicky(33) has investigated the pharmacological activity of peucedanin, pimpinellin and ostruthin. He found that they are almost insoluble in water, and that in solution or suspension they have no action on blood corpuscles, have no chloretic (choleretic?) action on mice and

and turtles and no diuretic action on mice. Ostruthin administered orally to mice is a mild purgative; dilute solutions of peucedanin and pimpinellin in frog perfusions produce slight contraction of the vascular system. Subcutaneous injection of these three substances in mice in large doses causes toxic effects, the greatest being with peucedanin and the least with pimpinellin. Wasicky also reported that intravenous injections in rabbits produces, in small doses, a slight rise in blood pressure and increased depth in breathing; large doses produce marked lowering of blood pressure and death. Simultaneous administration of peucedanin and pimpinellin with magnesium sulfate to mice leads to resorption of the latter so that with suitable doses, narcosis and eventual death occurs, while magnesium sulfate without the coumarins produces only mild purgative action.

Späth and Kuffner,(16) in carrying out a number of experiments on natural coumarins, found that they are highly effective in their action on the fresh-water fish, Lebistes reticulatus, in spite of the fact that most of these coumarins are only sparingly soluble in water. They found that initially these substances are strong stimulants, but then the action becomes moderate. Fish gradually lose their balance, and remain steady or swim on their backs; movement is then suspended and finally they die. They tested forty coumarins and found that many of them show a poisonous effect similar to picrotoxin.

Khellin, which is also known as visammin, has been found to have a specific local dilating action on the coronary vessels of the heart in animals and man. Clinical findings indicate that khellin has a specific action which brings relief from spastic pain of angina pectoris and other heart diseases. Bronchial asthma is also said to

be relieved by khellin by relaxation of the bronchial muscles.(2)

The extract of Ammi majus L. has been well known for ages and has been used in Egypt as a home remedy against leucodermia, a condition characterized by the absence of skin pigmentation.(12) The crude extract is toxic to both cold and warm blooded animals, but pure xanthotoxin, a constituent of the extract, is non-toxic to man in therapeutic doses.

Peucedanin has been found to possess the remarkable property of accelerating alcoholic fermentations(31) and the root from which it is obtained was formerly believed to have diuretic activity.

Pimpinellin, the crystalline principle obtained by alcoholic extraction from Pimpinella saxifraga L., has been used in medicine as a diuretic and diaphoretic and for a variety of complaints, but probably has no medicinal effect except as an aromatic.

The root of Peucedanum ostruthium Koch, from which no fewer than five coumarin derivatives have been isolated, was formerly used in a variety of complaints with so much supposed success as to have gained the title of divinum remedium. It is now regarded as merely a stimulant aromatic, but is not used in this country as a remedy. Its leaves are used as a pot herb and to flavor cheese.

BACKGROUND OF THE PROBLEM

Associate Professor of Pharmacognosy Tracey G. Call of the School of Pharmacy, Montana State University, considered the possibility that some of the many substances isolated by Späth might be present in the Umbelliferae of the western United States. During a field trip through northern Oregon in 1949, he made a collection of the roots of Pteryxia terebinthina (Hook.) Coult. & Rose var. terebinthina (Herbarium

Specimen No. 166). The plant was identified by Dr. Lincoln Constance, Curator of the Herbarium, University of California. Since a review of the literature revealed no investigation into its constituents, Professor Call undertook a preliminary study of this variety of Pteryxia terebinthina.

Professor Call isolated a crystalline substance from the roots of this plant in July 1949, and a preliminary investigation indicated that the material might be a coumarin derivative.

The immediate purposes of this study were:

1. Improvement of the method of isolation and purification of the crystalline substance from Pteryxia terebinthina (Hook.) Coult. & Rose var. terebinthina.
2. Determination of its physical characteristics.
3. Preliminary investigation of its chemical structure.

DESCRIPTION OF THE PLANT AND ROOTS

Pteryxia terebinthina (Hook.) Coult. & Rose var. terebinthina is an umbelliferous plant which has been known as Selinum terebinthinum Hook. Fl. Bor. Am. 1, 266, as syn. (1832); Laserpitium terebinthinum Dougl.: Hook. Fl. Bor. Am. 1, 266, as syn. (1832); Cymopterus terebinthinus T. & G. Fl. N. Am. 1, 624 (1840); and Pteryxia terebinthacea Nutt.; T. & G. Fl. N. Am. 1, 624, as syn. (1840)(8)

Mathias and Constance(8) describe the plant as follows:

> Plants caulescent to subcaulescent, 10-60 cm. high; leaves gray-green, ovate-oblong to broadly ovate in general outline, excluding the petioles 3-13 cm. long, 3-12 cm. broad, pinnately or ternate-pinnately decompound, the ultimate divisions linear to subcuneate, rigid, acute, 1-4 mm. long, about 1 mm. broad, more or less confluent; petioles 2-16 cm. long; peduncles 10-35 cm. long; involucel dimidiate, the bractlets

linear to rarely obovate, acute, entire or rarely toothed, 2-6 mm. long; 7-24, unequal 5-70 mm. long; pedicels 1-8 mm. long; flowers yellow; fruit ovoid to ovoid-oblong, 7-11 mm. long, 7-11 mm. broad, the wings undulate-crisped, equaling or exceeding the body, the dorsal equaling the lateral or rarely shorter; oil-tubes 3-12 in the intervals, 6-20 on the commissure and sometimes at the base of the wings.

TYPE LOCALITY: "Common on the sandy grounds of the Wallahwallah [Walla Walla] River, North-West coast of America," Washington, Douglas.

DISTRIBUTION: Columbia Plateau of eastern Washington and Oregon (Cotton 1080, Constance & Beetle 2688, Thompson 4778).

The roots are up to 5 cm. in diameter, tapering gradually. The diameter about 60 cm. from the surface is up to 2 cm. The crown in old roots is branched and the upper portions of the root bears many projecting leaf scars. The corky surface is brown and deeply fissured longitudinally. The cortex is tough and is easily peeled from a weak, porous phloem. The xylem is also weak. The phloem contains considerable amounts of oleoresins.

The roots were easily broken by hand into pieces 2 to 5 cm. in length. The pieces were spread out on newspaper and dried at room temperature and an average relative humidity of thirty per cent until the loss in weight over a twenty-four hour period was less than one-half of one per cent of the total weight. The dried roots were then milled in a Jacobson laboratory mill, Wiley mill or Waring blender to a fineness of #20 mesh.(3)

EXPERIMENTAL

Isolation and Recrystallization Methods

Isolation of the crystals. To 500 grams of the powdered root was added 1500 ml. of petroleum ether (30°-60° C.) in a four liter Erlenmeyer flask. The flask was then stoppered and shaken for a period of five minutes several times during the day. After twelve to twenty-four hours, the petroleum ether was filtered off, 600 ml. of solvent again added, and the process repeated until the root had been extracted with a total of four portions of solvent. The solvent from each portion was filtered into a separate beaker.

The yellow filtrate was allowed to evaporate in air without heating. As evaporation proceeded, a sticky, resinous material adhered to the side of the beaker. Yellowish crystals were deposited on the bottom of the beaker along with a yellow, resinous material. When the amount of the filtrate in the beaker was reduced to approximately one-tenth of the original volume, it was decanted to another beaker and the crystals were washed with several small portions of solvent. This procedure was followed for each portion of filtrate. The crystalline material and adhering resinous matter from each filtrate were then combined for recrystallization.

The first solvent extraction of the root usually yielded the most crystals and the least amount of resin, but as the extractions were repeated, more resin and fewer crystals were obtained.

Another method of isolating the crystals involved the extraction

of fifty grams of the ground root with four 200 ml. portions of petroleum ether (30°-60° C.) in a Waring blender. The process was satisfactory except that the yield was so small and the procedure so time-consuming that it was abandoned in favor of the technique described above.

Recrystallization methods. The impure crystals were dissolved in petroleum ether (30°-60° C.) by heating for five minutes in a one liter Erlenmeyer flask under reflux. The supernatant liquid was filtered through a funnel warmed by a hot water jacket. More solvent was then added to the flask and the process repeated until most of the crystals had been dissolved.

The combined filtrates were again allowed to evaporate spontaneously and when the volume had decreased to one-tenth the original volume, the solvent was decanted and the crystals washed with several portions of fresh solvent. This process of recrystallisation was repeated until colorless crystals were obtained. The crystals were then dried in a vacuum desiccator at 47° C. in the presence of paraffin and anhydrous calcium chloride.

Recrystallization of the extracted crystals using activated charcoal to remove resin and colored matter was abandoned because the loss of crystals to the adsorbent was prohibitive.

The addition of a few milliliters of alcohol to the petroleum ether solvent during recrystallization aided in separating most of the resinous matter present, but because the crystals themselves were so soluble in alcohol, there was considerable loss.

Recrystallization from various combinations of solvents such

as cyclohexane-petroleum ether, cyclohexane-diethyl ether, petroleum ether-diethyl ether and benzene-petroleum ether was not as successful as from petroleum ether alone. Water-alcohol combinations were also unsuccessful and almost invariably resulted in milky emulsions with no crystallization.

The yield of crystals after recrystallization from approximately 8000 grams of ground root was eleven grams or approximately 1.4%. Because of the small yield and the time-consuming methods of extraction and repeated recrystallization, characterization of the crystals was greatly handicapped.

Physical Constants

Melting point. The melting point of the crystals was not always consistent and varied from 77° C. to 82° C. (uncorrected), with a melting range of approximately two degrees. All melting point determinations were made using a Thiele tube with cottonseed oil and a Sargent general purpose thermometer.

Mixed melting points of the crystals of low melting range and high melting range resulted in a melting point between the two extremes, indicating a variation in degree of purity. Efforts to recrystallize the low melting crystals were discontinued because of the loss of material from an already small yield.

Optical activity. A Kern Full-Circle polarimeter manufactured by Kern & Co., Ltd., Aarau, Switzerland was used to determine optical activity of the crystals. This determination was carried out in an air-conditioned room at a constant temperature of 20° C.

0.225 gram of crystals was dissolved in 50 ml. of 95% ethyl alcohol in a volumetric flask and carefully filtered. The two decimeter tube of the polarimeter was filled with this solution and from an observed average rotation of positive 0.0381, the specific rotation was calculated to be a positive 4.22°. Since this degree of specific rotation is so small and because of the possibility of experimental error in determining an observed rotation of only 0.0381°, it is probable that the crystalline material is optically inactive.

Qualitative Tests for Elements

The tests for the elements nitrogen, sulfur and the halogens were made in the manner described by Shriner and Fuson[18] in which their detection depends on converting these elements into water-soluble ionic compounds by sodium fusion and then applying specific tests for each element. All the tests were negative, indicating the presence of carbon, hydrogen and oxygen only.

Solubility Tests and Solubility Classes

The crystals were insoluble in cold or hot water, but soluble in most organic solvents such as benzene, chloroform, diethyl ether, alcohol, cyclohexane and carbon tetrachloride. They were only slightly soluble in hot petroleum ether (30-60° C.).

To assist in assigning the crystalline material to a general class of compounds, the solubility classification tests described by Shriner and Fuson[18] was used. This testing procedure consists of determining the solubility of 0.1 gram of the finely powdered substance in three milliliters of solvent and assigning the compound to a subdivision by reference to its solubility.

The crystals were insoluble in water, cold 5% sodium hydroxide and 5% hydrochloric acid, but were soluble in concentrated sulfuric acid with blue fluorescence and only slightly soluble in syrupy phosphoric acid. Although the crystals were practically insoluble in 5% sodium hydroxide, the solution turned yellow. These solubility tests indicated a neutral, carbonyl compound of more than nine carbons.

Tests for Functional Groups[18]

Unsaturation. The compound did not decolorize bromine in carbon tetrachloride but this is not conclusive evidence of saturation since not all olefinic compounds take up bromine and the presence of negative groups on the carbon atoms of ethylenic bonds causes the addition of bromine to be slow or completely inhibited.

Using alcohol-free, neutral acetone as a solvent for the crystals, 2% aqueous potassium permanganate was reduced after three to four minutes. 112 mg. of the crystals reduced approximately 150 ml. of 0.1173 N potassium permanganate added dropwise from a burette.

Alcohol and phenol groups. The Schotten-Baumann Reaction was negative. This reaction depends upon the reactivity of an alcohol or phenol group with benzoyl chloride in an alkaline solution to precipitate an ester. Since no reaction occurred, it is probable that the substance does not possess a reactive alcohol or phenol group.

Alkoxyl groups. The crystalline material did not respond to Zeisel's Alkoxyl test in which hydriodic acid is used to cleave functional groups containing methyl, ethyl, n-propyl or isopropyl radicals attached to oxygen. This would indicate that if any alkoxyl groups are present in the substance they must be n-butyl or larger

since such groups are difficult to cleave, and the iodide formed is too high boiling to be volatilized and thus react with the test medium.

Carbonyl groups. A few crystals of the compound, dissolved in a minimum of ethyl alcohol, reacted with 2,4-dinitrophenylhydrazine to produce a cloudy solution but not a precipitate. A reddish-orange, resin-like material separated and adhered to the sides of the test tube. This test indicates the probable presence of a carbonyl group. The compound would not reduce Tollen's reagent or react with Schiff's reagent, indicating that the carbonyl group present was a ketone and not an aldehyde.

Phenolic groups. A saturated aqueous solution and an alcoholic solution of the compound gave no coloration with ferric chloride test solution. Since many phenols and enols do not give a positive test with ferric chloride the test cannot be considered conclusive.

Ferric hexathiocyanatoferriate test. The compound did not respond to this test. Compounds containing oxygen usually produce a positive test with this reagent by dissolving the red complex salt and producing a red solution. However, saturated, unsaturated and aromatic hydrocarbons and a few high-molecular weight ethers give negative tests, so this test was also inconclusive.

Hydroxamic acid test for esters and anhydrides. Vogel[32] describes a test for esters and anhydrides in which they produce a characteristic color after reacting with hydroxamic acid and adding ferric chloride. The substance gave a negative test.

Molisch carbohydrate reaction.[11] The crystals did not respond to the Molisch carbohydrate test.

Saponification Equivalents

Alcoholic-potassium hydroxide method. To 0.2326 gram and 0.1925 gram respectively, each in a 125 ml. flask, was added 15 ml. of approximately 0.25 N alcoholic potassium hydroxide solution. A blank containing 15 ml. of the hydroxide solution was also prepared and the three flasks gently boiled under reflux for a period of one hour and twenty minutes and the flasks then cooled. Two drops of phenolphthalein indicator solution was then added to each flask and each was then titrated to the phenolphthalein end point with 0.25 N hydrochloric acid. The blank required 12.5 ml. of acid, the 0.2326 gram sample required 6.1 ml. and the 0.1925 gram sample required 6.9 ml. of acid to neutralization. From the above data, neutralization equivalents of 145.37 and 137.5 were calculated.

The end point of the neutralization was somewhat difficult to observe since the solutions became a bright yellow on the addition of alkali and this color interferred with an accurate observation of the end point.

Diethylene glycol-potassium hydroxide method. To 0.4259 gram and 0.5382 gram of the compound, each in a 125 ml. flask, was added 10 ml. of 0.7850 N potassium hydroxide in diethylene glycol reagent and each mixed thoroughly, stoppered and heated in an oil bath to a temperature of 80° C. for three minutes, then removed from the heating bath, shaken vigorously, and heated again to 125° C. for three minutes. After cooling, the stoppers were removed and washed with distilled water so that the rinsings drained into the flask. Fifteen ml. of distilled water was then added to each flask, the contents thoroughly mixed and

titrated with 0.2593 N hydrochloric acid using phenolphthalein as the indicator. The 0.4259 gram sample required 18.45 ml. of acid and the 0.5382 gram sample required 14.9 ml. to the end point. From the above data, saponification equivalents of 135.65 and 134.88 were calculated.

It was noted that after neutralization was obtained and the flasks were allowed to stand for several minutes, the solutions again became basic as shown by the change in color of the indicator. Over a period of four days, the saponifications carried out with diethylene glycol required approximately 3 ml. more acid to the end point. This was not pursued further. The saponification equivalents reported above are those determined immediately after the reaction.

Dodge[10] describes a similar phenomenon in his experiments on quantitative hydrolysis of coumarin with alkaline hydroxides. He found after heating 1.05 grams of coumarin one hour at 100° C. with 26.3 ml. of 0.5 N alcoholic potassium hydroxide and adding 25 ml. of water, that the amount of 0.5 N hydrochloric acid required to neutralize the excess alkali varied from 10.9 ml. when titrated immediately to 24.2 ml. after 162 hours. He observed a similar phenomenon with limettin (also known as citropten), a dimethoxycoumarin obtained from the sediment which is deposited from expressed oil of limes and also in the ester determination of oil of bergamot which is an expressed oil and contains 5-6% of non-volatile matter in which is present the crystalline bergaptene, a coumarin derivative. He explains this phenomenon as probably due to the fact that the tendency to lactone formation is opposed to the hydrolysis and that a condition of equilibrium between the lactone and alkaline salt is to be expected.

Action of Alkali

As previously mentioned in the solubility tests and the saponification reactions, the action of both aqueous and alcoholic alkali produced a yellow solution. Wawzonek[34] and Späth[28] state that the initial action of alkali on coumarins is the opening of the pyrone ring and the formation of the yellow alkali coumarinate. This appears to indicate the presence of a lactone ring in the chemical structure of the compound. Acidification of the alkali coumarinate of simple coumarins as a rule regenerates the original coumarin,[34] but this compound does not appear to be a simple coumarin since acidification did not change the color of the yellow solution which would indicate regeneration of the original compound.

Carbon-Hydrogen Determination

Microanalytical carbon-hydrogen determination[5] of a sample of the crystals (m.p. 80°-81° C.) indicated the following:

Carbon	65.87%
Hydrogen	5.98
Oxygen	28.15

The percentage of oxygen is the difference between the percentage of carbon and hydrogen and 100 since no other element was found to be present in the compound.

Molecular Weight Determination

Two molecular weight determinations by the Beckman cryscopic method, using benzene as the solvent, gave molecular weights of 299 and 311. Using the Rast method with exaltone (cyclopentadecanone) as the solvent, molecular weights of 370, 374, 405 and 407 were found. On the

basis of an average saponification equivalent of 136 (discarding the saponification equivalent of 145.37) and assuming that the compound possesses three ester groups or replaceable hydrogens, a molecular weight of 3 x 136 or 408 would be in fair agreement with the results of 405 and 407 found by the Rast method.

Based on the carbon-hydrogen determination and molecular weight determination, the empirical formula $C_{22}H_{24}O_7$ and molecular weight of 400.4 is postulated:

	%C	%H	%O
Found	65.87	5.98	28.15
Calculated for $C_{22}H_{24}O_7$ . . .	65.95	6.04	27.97

Degradation Reactions

Späth, Platzer and Schmid[27] describe a technique which they used for cleaving the coumarin athamantin to oroselon and isovaleric acid. This procedure was used in an effort to cleave the compound and to attempt an identification of the products. In the original literature, concentrated hydrochloric acid was used in the initial reaction, but because of an error in translation from the German to English, this reaction was carried out with fuming sulfuric acid instead.

Four grams of air dried, unrecrystallized crystals from the petroleum ether extract was added to 50 ml. of absolute methyl alcohol in a 250 ml. Erlenmeyer flask, followed by 25 ml. of 15% fuming sulfuric acid. The mixture was shaken and then heated under reflux for thirty minutes. The very dark mixture was then cooled and steam distilled to drive over any volatile acid into a 400 ml. beaker containing 10 grams of potassium hydroxide in 50 ml. of absolute methyl alcohol. After

driving over 300 ml. of the liquid, the distillation was stopped. The distillate became yellow in the alkaline alcoholic solution and had a rather pleasant ester-like odor. This was gently boiled under reflux for one hour, the solution was then cooled in ice and acidified carefully to litmus with concentrated hydrochloric acid. The free acid, which had an unpleasant odor common to the four to seven carbon acids, was then steam distilled once more until 300 ml. total volume had been driven over. The aqueous distillate was then made basic with 10% sodium hydroxide solution and evaporated on a steam bath to a volume of about 25 ml. This solution was again cooled in ice, acidified with dilute hydrochloric acid and extracted in a separatory funnel with four 15 ml. portions of ethyl ether. The ether extract was dried over anhydrous magnesium sulfate for forty-eight hours. The ether extract was then filtered into a small suction flask and the flask was stoppered and attached to a water pump. The flask was heated with warm water until all but 2 or 3 ml. of ether had been driven off. The solution was then transferred to a three inch test tube to which had been fitted a small glass tube as a condenser, the remainder of the ether driven off, and about one ml. of a reddish-brown liquid residue remained. This was distilled into another small, water cooled test tube by heating in a silicone bath. Approximately 0.5 ml. of colorless, slightly viscous, water insoluble acid was driven over which retained the unpleasant odor first encountered in the initial acidification.

To determine the boiling point of this liquid, a small capillary tube was sealed about 5 mm. from one end and placed in the test tube which contained the acid. The test tube was attached to a thermometer in an oil bath. The temperature was raised until a rapid flow of

bubbles came out of the capillary tube and passed into the liquid. The bath was then allowed to cool while stirring and the temperature noted when the bubbles ceased to come out of the capillary tube. The boiling point was found to be 182°-183° C. (uncorrected).

The refractive index, using an Abbe refractometer, was 1.4545 at 20° C.

A congealing point determination was made by immersing the test tube which contained the acid into an ice bath and while stirring the bath constantly, noting the temperature at which the first crystals appeared. The congealing point was found to be 11°-11.25° C.

An anilide derivative of the acid was prepared by refluxing it with 1 ml. of thionyl chloride in a test tube for thirty minutes. The mixture was cooled and a solution of 1 ml. of redistilled aniline in 15 ml. of benzene was added and the mixture warmed on a steam bath for two minutes. An amorphous precipitate formed. The benzene solution was separated by centrifuging, and then decanted into a separatory funnel and washed successively with 1 ml. of water, 2.5 ml. of 5% hydrochloric acid, 2 ml. of 5% sodium hydroxide and finally with 1 ml. of water. The benzene was evaporated and the anilide recrystallized with some difficulty by dissolving it in a minimum of ethyl alcohol, heating it gently on a steam bath and then adding water dropwise until just cloudy; a drop or two more of alcohol was added to clear the solution and crystallization induced by cooling in an ice bath and diligently scratching the sides of the container with a glass rod for a period of fifteen minutes.

After drying in a vacuum desiccator over calcium chloride,

the melting point of the derivative was 74.5°-75.5° C. After one more recrystallization, the melting point was 77°-78° C.

The literature reveals that the anilides of n-dodecanoic (lauric) acid, tiglic (cis-α-methylcrotonic) acid and 4-methyl hexanoic acid-1 (γ-methyl-n-caproic) acid give anilides which melt in the range of 76.5° to 78° C.

Lauranilide . . . . . 78° C.[14] and 76.5° C.[4]

Tiglanilide . . . . . 77° C.[11]

γ-methyl-n-caproanilide . . 76.5° C.[11]

However, n-dodecanoic acid is a solid acid and melts at 43.2° C. which is not in agreement with either the physical state or melting point of the acid found. Tiglic acid is a water soluble, crystalline substance which melts at 64.5° to 65° C., also not in agreement with the isolated acid. 4-methyl hexanoic acid-1 is a liquid, water insoluble acid which boils at 217°-218° C. and has a refractive index of 1.4211 which does not agree with the characteristics of the unknown acid.

The empirical formulas of $C_{18}H_{30}NO$ for the anilide of n-dodecanoic acid, $C_{14}H_{11}NO$ for the anilide of tiglic acid and $C_{13}H_{20}NO$ for the anilide of 4-methyl hexanoic acid-1 do not agree with the carbon-hydrogen determination[6] of the unknown acid:

	%C	%H
Found for anilide of unknown acid . . .	68.06	6.92
Calculated for $C_{18}H_{30}NO$	78.20	10.94
Calculated for $C_{14}H_{11}NO$	74.96	8.00
Calculated for $C_{13}H_{20}NO$	75.68	9.77

This would indicate that the unknown acid was probably not n-dodecanoic, tiglic or 4-methyl hexanoic acid-1 and that more investigation will be required to determine its structure.

Two grams of the purified crystalline material (m.p. 78°-79° C.) was cleaved in the same manner described above, except that concentrated hydrochloric acid was used instead of fuming sulfuric acid as in the initial reaction. Since the crystals used in this reaction were free of any resinous material and the acid used was less reactive, the reaction was observed without the complication of darkening of the solution which had appeared previously. During the initial heating under reflux with the acid and alcohol, a brown, oily, immiscible layer separated which did not distil over with the unknown acid during the steam distillation. On cooling, this immiscible material congealed and separated from the rest of the solution. This acidic solution was filtered, and when a few milliliters of 20% sodium hydroxide was added to a portion of the filtrate, the solution became yellow, which indicated the possible presence of a lactone. The solution was extracted with several small portions of ether in a separatory funnel and dried over anhydrous sodium sulfate for several days, then filtered and the ether evaporated. A light yellow substance , which had an odor like that of burned sugar, appeared in the bottom of the flask. This was warmed on a steam bath with a little water to which had been added a few drops of alcohol. Some gummy globules separated during the heating, so the solution was filtered and the filtrate again reheated on the steam bath with the addition of a few drops of water until just cloudy. This was then cooled in an ice bath with scratching until a light tan, amorphous

material separated, which after drying, weighed 0.13 grams and melted at 177°-178° C. This amorphous material was insoluble in cold water, insoluble in 5% sodium bicarbonate solution, but soluble in 5% sodium hydroxide solution which became yellow and showed a green fluorescence under ultraviolet light. The solubility data indicated that it may possibly be a weak acid.

The unknown acid which was separated by steam distillation could not be investigated further because of the insignificant yield, but by its odor, it appeared to be the same as the unknown acid mentioned previously.

Shah and Shah[17] describe a modified method of converting coumarin derivatives into o-methoxycinnamic acids as an aid in identifying an unknown coumarin. This method was carried out as follows: One gram of the crystalline substance was dissolved in a minimum of acetone and 10 ml. of dimethyl sulfate was added, followed by 25 ml. of 20% potassium hydroxide solution. The mixture was heated to 100° C. and more alkali and dimethyl sulfate (15 ml. in all) was added from time to time for an hour, keeping the mixture alkaline. After cooling, the mixture was acidified with hydrochloric acid and a flocculent precipitate separated. The mixture was extracted with several small portions of ether and the ether evaporated off. The yellow, amorphous residue was recrystallized by dissolving it in a minimum of alcohol and cooling in an ice bath. However, a gummy substance again separated which interferred with crystallization during the first several attemps, so crystallization did not occur until this gummy, resinous matter was removed with a spatula. 0.12 gram was recovered after drying, melting

point 163°-165° C. It was insoluble in cold water, soluble in 5% sodium bicarbonate, did not discolor ferric chloride solution, but did decolorize 2% potassium permanganate solution after it had been dissolved in alcohol-free, neutral acetone. No final conclusion is possible as to the chemical nature of this product because the small yield made further investigation impossible.

Dermer and King[9] were successful in identifying the acyl group in many esters by a modified method of preparing N-benzylamides. By this method, a derivative is prepared directly from the ester by refluxing with benzylamine in the presence of a salt catalyst. One gram of the crystalline material was added to 3 ml. of benzylamine together with 0.1 gram of ammonium chloride. The mixture was refluxed for an hour in a Pyrex test tube attached to a reflux condenser. On cooling, an unpurifiable gum resulted which could not be separated. The procedure was then repeated after first subjecting the crystalline material to preliminary methanolysis in a solution of 0.1 gram of sodium in 5 ml. of absolute methyl alcohol under reflux for one-half hour. This method was also unsuccessful as an oil separated.

Dermer and King report that coumarin was one of the esters which gave only an unpurifiable gum or oil with this procedure, which may account for the failure to get a useful derivative in this instance.

By following a procedure which Späth and Christiani[21] used to saponify the furanocoumarin ostruthol to angelic acid and oxypeucedanin hydrate, two products were obtained as follows: 1.5 gm. of the crystalline material was heated under reflux with a solution of 4.5 gm. of potassium hydroxide in 60 ml. of absolute methyl alcohol for 30

minutes, then diluted with 20 ml. of distilled water and the methyl alcohol was distilled off in vacuum. The solution was then acidified with hydrochloric acid. When the acid was added, the solution became milky and a brown, resinous material separated. After one hour a white precipitate settled out and on standing overnight the brown, resinous fraction also became solid and precipitated out of the solution. The precipitate was filtered with suction and recrystallized by dissolving it in a minimum of methyl alcohol on a steam bath and adding water dropwise until the solution became cloudy. Crystallization was induced by cooling the solution in ice and scratching the sides of the container with a glass rod. After drying over calcium chloride, the precipitate weighed 0.1 gram and melted at 160°-161° C. A small amount added to an alcoholic solution of potassium hydroxide turned the solution yellow, which would indicate the probable presence of a lactone group. The compound did not discolor ferric chloride test solution.

Carbon-hydrogen microanalysis[7] of the precipitate indicated 64.89% carbon, 5.73% hydrogen and 29.38% oxygen.

By the Rast method, using exaltone (cyclopentadecanone) as the solvent, molecular weights of 249, 256 and 263 were found.

On the basis of the carbon-hydrogen determination the formulas $C_{12}H_{13}O_4$ (molecular weight 221.22) and $C_{14}H_{15}O_5$ (molecular weight 263.26) compare as follows:

	%C	%H	%O
Found	64.89	5.73	29.38
Calculated for $C_{12}H_{13}O_4$	65.15	5.92	28.93
Calculated for $C_{14}H_{15}O_5$	63.87	5.75	30.39

The formula $C_{12}H_{13}O_4$ is in closer agreement to the analytical data for carbon and hydrogen, but the formula $C_{14}H_{15}O_5$ is in better agreement with the molecualr weight determinations. This will require more investigation to determine the actual composition.

The filtrate from the initial reaction after acidification was saturated with sodium chloride and exhaustively extracted with five 25 ml. portions of ether and the extract was distilled at 15 mm and 115° C. oil bath temperature. Approximately 0.1 ml. of a liquid was collected in a small test tube. Further heating of the flask with a direct flame at the same reduced pressure yielded approximately 0.1 ml. more. Colorless, needle-shaped crystals collected on the sides of the receiver during the distillation. The distillate was again dissolved in a small amount of ether and redistilled at 8 mm pressure and 110°-150° C. The very small yield of crystals melted at 28° C. An attempt to identify this compound as an acid by the preparation of an amide was unsuccessful and the compound could not be characterized further.

SUMMARY AND CONCLUSIONS

1. A method has been developed for isolating and recrystallizing a crystalline substance from the roots of Pteryxia terebinthina (Hook.) Coult. & Rose var. terebinthina.
2. The preliminary physical and chemical characteristics of this crystalline substance have been determined.
   a. The physical constants have been determined.
   b. The formula $C_{22}H_{24}O_7$ has been postulated for the compound.

c. The reaction of the compound with alkali indicates the presence of a lactone group.

d. The reaction of the compound with 2,4-dinitrophenylhydrazine indicates a carbonyl group.

e. The compound reduces potassium permanganate.

3. An unidentified acid has been cleaved from the compound.

4. The reactions of the compound indicate that it may be a coumarin derivative.

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